

Award Number: W81XWH-05-1-0261

TITLE: Breast Cancer Research Program (BCRP) - Predoctoral Traineeship -
Elucidating the Role of the Type III Transforming Growth Factor-beta Receptor in Bone
Morphogenetic Signaling in Breast Cancer

PRINCIPAL INVESTIGATOR: Kellye Kirkbride

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27710

REPORT DATE: March 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-03-2008		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 1 MAR 2005 - 29 FEB 2008	
4. TITLE AND SUBTITLE Breast Cancer Research Program (BCRP)- Predoctoral Traineeship – “Elucidating the Role of the Type III Transforming Growth Factor-beta Receptor in Bone Morphogenetic Signaling in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0261	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Kellye Kirkbride E-Mail: kkirkbri@gmail.com				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Durham, NC 27710				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We have identified and characterized the Transforming Growth Factor- β (TGF- β) Type III Receptor(T β RIII) as a cell surface receptor for the Bone Morphogenetic Proteins(BMP). This report demonstrates that loss of T β RIII results in alterations in intracellular signaling by BMP, specifically phosphorylation of the intracellular effector, Smad1. We determine that the effect of loss of T β RIII on Smad1 phosphorylation is cell-type specific. We have also determined that T β RIII facilitates internalization of ALK6 in the presence of β -arrestin2 (β -arr2). Here we demonstrate that T β RIII and β -arr2 together dramatically enhance the immunoprecipitation of a complex containing ALK6. We also show that this complex of T β RIII, β -arr2 and ALK6 dramatically increase BMP-2-induced transcriptional responses. We have begun to apply this molecular data to elucidate the role of T β RIII and BMP proteins in carcinogenesis to better understand their potential role in breast cancer. Recent data demonstrates an increase in expression of BMP ligands by a number of cancer cell types. Here we demonstrate that BMP treatment decreases expression of T β RIII and induces an epithelial to mesenchymal transition (EMT) in pancreatic cancer, suggesting a mechanism by which T β RIII is lost during carcinogenesis and leads to an increase in invasiveness of cancer cells.					
15. SUBJECT TERMS No subject terms provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER (include area code)

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Introduction:

The TGF- β Type III Receptor (T β RIII) is the most abundant receptor for one of the largest families of pleiotrophic growth factors, the Transforming Growth Factor- β (TGF- β) superfamily [1]. T β RIII has been characterized as a cell surface receptor for all three isoforms of TGF- β [2] and superfamily member, inhibin [3]. Based on structural homology, we sought to determine whether T β RIII is able to bind members of the largest subfamily of this superfamily, the Bone Morphogenetic Protein (BMP)/Growth Differentiation Factor (GDF) subfamily.

In recent years, our lab has demonstrated that T β RIII is frequently lost, through loss-of-heterozygosity and DNA methylation, in a number of cancer cell types, including breast, lung, ovarian, pancreatic, and prostate cancers [4-8]. Restored expression of T β RIII in these cancer cells resulted in an inhibition of migration and invasion. The contribution of BMP signaling in cancer (including breast, colon, lung, pancreatic and prostate cancers) is beginning to be elucidated and has been demonstrated to be heavily cell-type specific [9-12]. BMP signaling has been linked to proliferation, along with the migration and invasion of breast cancer cells [10, 13, 14].

Alteration of cell surface expression can alter downstream signaling and cellular responses to extracellular ligands. BMP initiates signaling by binding to the high affinity type I cell surface receptors, ALK3 or ALK6, which then complex with and are activated by a type II receptor (BMPRII or ActRII). Active ALK3 and ALK6 may then phosphorylate the ubiquitous downstream effector protein, Smad1, which complexes with Smad4 to accumulate in the nucleus and mediate target gene transcription [15-17]. To date, very little is known about the regulation of the BMP receptors- including mechanism for internalization and degradation. Internalization of BMP receptors is constitutive and necessary for prolonged activation of BMP signaling [18]. At the outset of these studies, we **hypothesized** that **T β RIII is able to mediate BMP signaling through its ability to bind BMP and form a stable cell surface complex with the BMP type I receptors, ALK3 and ALK6.** We have since modified this hypothesis to state that we believe that T β RIII enhances a **stable internal receptor complex.**

In the previous annual summaries, I have shown that T β RIII binds multiple members of the BMP subfamily, including BMP-2, BMP-4, BMP-7 and GDF-5. In addition, I determined that there are 2 ligand binding sites on T β RIII for BMP and that the affinity of T β RIII for BMP is on the same order of magnitude as that for TGF- β 1. The increased expression of T β RIII increased BMP binding to the BMP receptors, increased BMP-2-mediated transcriptional responses and may be the consequence of differential internalization of ALK3 and ALK6 mediated by T β RIII.

Since the last report, I have more closely examined the role of T β RIII and β -arrestin2 (β -arr2) in enhancing internalization of ALK6 and its affect on BMP signaling. I have also determined a role for loss and restored expression of T β RIII in BMP-mediated signaling and cellular responses in cancer cell models to begin to elucidate the role of BMP in breast cancer. At this time, I am pleased to report the progress that has been made focused on these goals and discuss future projects generated as a result of these data.

Results/Discussion:

This past year I have made significant progress toward better understanding the molecular role of T β RIII in BMP signaling in cell biology. Understanding the interaction between T β RIII and BMP-mediated signaling will aid in our understanding of the role of

the loss of T β RIII in breast carcinogenesis. Like any good research project, we have just begun to scratch the surface of understanding the role of T β RIII in both BMP signaling and in breast cancer. During the course of these studies, I have collaborated with other students and postdoctoral fellows using systems they have optimized to work more efficiently towards a better understand of how T β RIII affects BMP signaling. To do this, it has required that we use not only breast cancer cells, but additional cell types with the goal of better understanding the molecular mechanisms of T β RIII action so that we can apply these findings to better understanding breast cancer. As a consequence, these studies have lead to many more questions than answers and Dr. Gerard Blobe's lab is actively pursuing these answers in regards to breast cancer.

I. T β RIII is essential for BMP-mediated EndMT and loss of T β RIII decreases BMP-sensitivity.

In previous years, I demonstrated that increasing expression of T β RIII acts to increase that affinity of the BMP signaling receptors, ALK3 and ALK6, for ligand and increase transcriptional response downstream. To date, there are two major defined functions of T β RIII RIII in cell biology, including (1) being essential for an endothelial to mesenchymal transition (EndMT) in avian ventricular endothelial cells [19] and (2) the inhibition of cell migration and invasion [4]. To test the physiological relevance of T β RIII in BMP signaling, we examined whether T β RIII is essential for BMP-induced EndMT. This past year, in collaboration with another graduate student Todd Townsend in Dr. Joey Barnett's Lab at Vanderbilt University, we have demonstrated that T β RIII expression confers the ability of BMP-2 to induce EndMT in avian ventricular endothelial cells (**Appendix A, Figure 7**) [19]. This effect was unique to BMP-2 and not BMP-4, BMP-7 or GDF-5. These data suggest that while we have biochemically characterized T β RIII as a receptor for BMP and uncovered a number of significant functions for T β RIII in BMP signaling, more detailed molecular studies into the role of T β RIII in BMP signaling and cell biology are warranted. This molecular information can now be applied to established models of epithelial to mesenchymal transistion (EMT) in breast cancer, which is being pursued by another graduate student in the lab, to understand the role of BMP and T β RIII in breast cancer.

II. Dichotamous effect of T β RIII in cancer cells

Since these experiments were an increase in T β RIII expression, we wanted to determine the affect of the loss of T β RIII on BMP-responsiveness. T β RIII levels were decreased using short-hairpin RNA (shRNA), in several different cell lines. The loss of T β RIII resulted in a decrease in BMP responsiveness in the prostate cancer cell line, PC-3 (**Appendix A, Figure 6**). The loss of T β RIII resulted in a shift of BMP responsiveness, meaning higher concentration of BMP-2 were required to significantly increase Smad1 phosphorylation, than cells with normal T β RIII expression.

Of note, the effect of knocking down T β RIII in cancer cells was highly cell-type specific, as loss of T β RIII had no effect on MDA-MB-231 breast cancer cells responsiveness to BMP and had an opposing effect on Smad1 phosphorylation in the pancreatic cancer cell model, Panc-1 (**data not shown**). This data emphasize that T β RIII's role in BMP signaling is likely to be cell-type specific. While these findings were initially disappointing in terms of breast cancer, they are not entirely surprising in that many of the initial studies examining the effect of T β RIII in TGF- β signaling initially concluded that

TβRIII was not essential for TGF-β signaling [20]. We realize the importance of examining the effect of loss of TβRIII on BMP signaling in a larger subset of breast cancer cells, in addition to other cancer cell types, and those studies are ongoing. The use of additional cancer cell lines in addition to breast cancer cells is important for understanding the broader impact of this ligand-receptor interaction on a wide range of cancer cell types. Our lab has recently demonstrated the importance of TβRIII not only in breast cancer, but also a number of other cancer cell types including lung, pancreatic, prostate and ovarian cancer [4-8]. If TβRIII is to be legitimized as a biomarker or potential therapeutic target for breast cancer, it is important for us to understand its effects in many breast cancer cell types with different genetic backgrounds. The fact that we did not see an effect in MDA-MB-231 suggest that it is important to more closely examine and determine the subset of cells that TβRIII loss most significantly affects in terms of TGF-β and BMP signaling. Future studies in Dr. Gerard Blobe's laboratory will be important to understand what aspect of BMP signaling is affected by loss of TβRIII in breast cancer. Furthermore, not all breast cancers lose TβRIII expression, so understanding the effect of maintained TβRIII on BMP signaling in these cancer cells is also of importance since a number of BMP family members are up-regulated in breast cancer.

There are a number of potential explanations worth further examination to explain the lack of an effect of loss of TβRIII on Smad1 signaling in MDA-MB-231 cells. Due to time constraints, we only examined the effect of loss of TβRIII on Smad1 phosphorylation; however, BMP have been shown to activate additional signaling cascades, including p38 MAPK, ERK and JNK. The effect of TβRIII on BMP-induced activation of these pathways remains to be investigated, so it remains possible that loss of TβRIII in breast cancer cells may alter these signaling cascades. In addition, further examination of whether MDA-MB-231 cells have alterations in the BMP signaling receptors should be determined. It is possible that BMP may bypass the need for TβRIII in signaling by constitutively activating the receptor complex downstream.

An alternative explanation is that this effect may be due to the complement of BMP receptors expressed by each cell type. Our present data would suggest that BMP signaling mediated by ALK6 is more dependent on TβRIII than ALK3 signaling and that this may explain why some breast cancer cells are more or less responsive to BMP and loss of TβRIII but warrants further investigation. Understanding these molecular mechanisms will be critical if we are to develop effective chemotherapies for TβRIII-positive breast cancers. These studies demonstrate that while we have made significant progress toward characterizing TβRIII as a BMP cell surface receptor, there is still a significant amount of work to be done to fully understand the dynamics of these ligand-receptor pair in breast cancer cell biology.

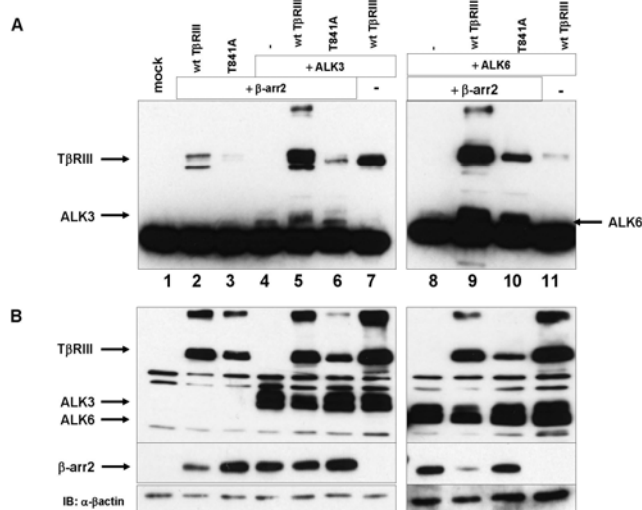


Figure 1. TβRIII facilitates complex formation between ALK6 and β-arr2. HEK293 cells were transiently transfected with HA-tagged TβRIII, ALK3, or ALK6 and FLAG-β-arr2. **A.** Cell lysates were immunoprecipitated with FLAG-β-arr2, followed by immunoblotting for HA. **B.** Total cell lysates were immunoblotted for the indicated constructs to verify expression.

III. TβRIII mediates complex formation between ALK6 and β-arr2.

Last year, I demonstrated that TβRIII mediates the differential internalization of the BMP type I receptors, ALK3 and ALK6. This year, we worked to better understand the effect of this differential increase on BMP-mediated signaling and the mechanism by which TβRIII facilitates ALK6 internalization. Previous data generated in our lab demonstrated that β-arr2 binds to and facilitates the internalization of TβRIII and leads to removal of the TGF-β type I

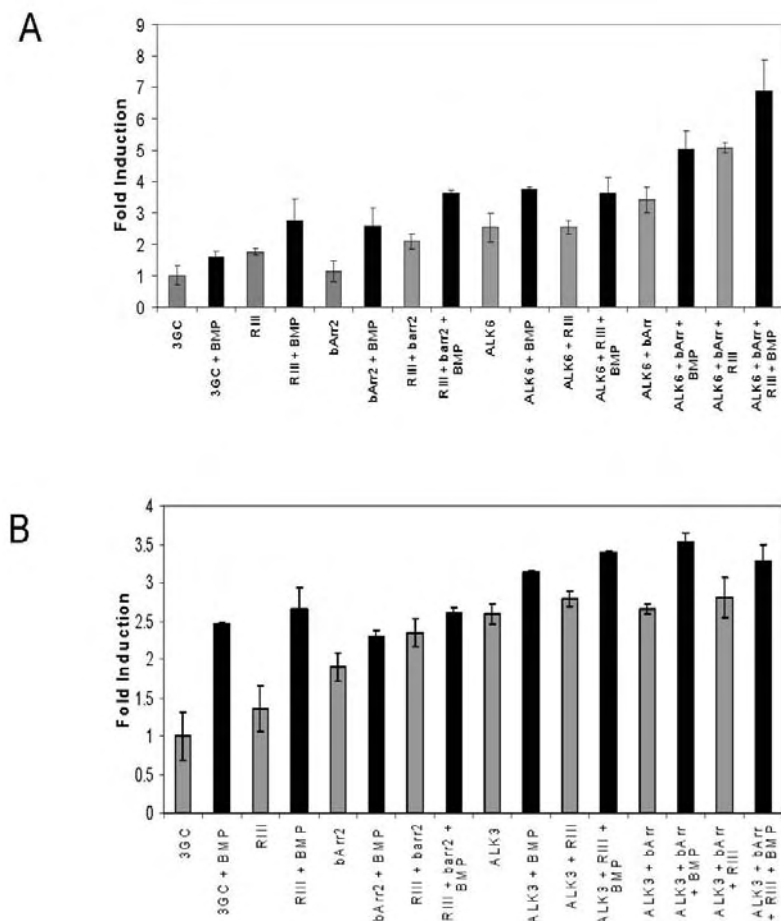
and type II receptors from the cell surface [21]. Therefore, we wanted to determine whether TβRIII alters that subcellular localization of ALK6 through a β-arr2-mediated mechanism. I was able to show that the expression of β-arr2 and TβRIII dramatically increased the association of

ALK6 with β-arr2, suggesting that TβRIII does indeed facilitate a complex that mediates internalization of ALK6 (**Figure 1**).

IV. TβRIII and β-arr2 increase signaling downstream of ALK6.

Next, we wanted to determine the effect of TβRIII and associated β-arr2 on BMP-mediated transcriptional responses. We used a BMP-responsive luciferase construct in P19 cells. The expression of TβRIII and β-arr2 acted synergistically to dramatically enhance BMP-responsiveness in combination to ALK6, but not ALK3 (**Figure 2** (last bars on the right, top and bottom respectively)). These data suggest that TβRIII and β-arr2 work in combination to internalize ALK6 and mediated BMP-

Figure 2. TβRIII and β-arr2 increase ALK6-mediated signaling. P19 cells were transiently transfected with the BMP-responsive luciferase reporter, 3GC2, with a combination of TβRIII, β-arr2, ALK6 (**A**) or ALK3 (**B**) as indicated. Luciferase readings were normalized to readings from a Renilla transfection control and to samples



responsiveness.

V. BMP induces increases in EMT-associated increases in invasion of cancer cells.

Another graduate student in the lab, Kelly Gordon and I, blended our two thesis projects to begin to determine the effect of BMP family ligands in cancer cell biology. By working together, we were able to dramatically expand our understanding of the role of T β RIII and BMP in cancer, which yielded valuable insight into how T β RIII may function in breast cancer biology. A role for BMP in carcinogenesis is an emerging field with very little known to date. In the chick endothelial cell studies, we determined that T β RIII is essential for BMP-induced EndMT (**Appendix A, Figure 7**). In addition, BMP has been shown to induce morphological changes and increases in migration of pancreatic cancer cells, consistent with an epithelial to mesenchymal transition (EMT) [22]. Kelly and I determined that multiple BMP family members that are able to bind T β RIII, including BMP-2, BMP-4 and BMP-7, are able to induce EMT in pancreatic cancer cells (**data not shown**). Next, we determined that BMP facilitated a loss of T β RIII expression in these cells (**data not shown**). This loss of T β RIII was at both the mRNA and protein level, suggesting that an increase in BMP ligands during carcinogenesis is a mechanism by which cancer cells may downregulate T β RIII expression.

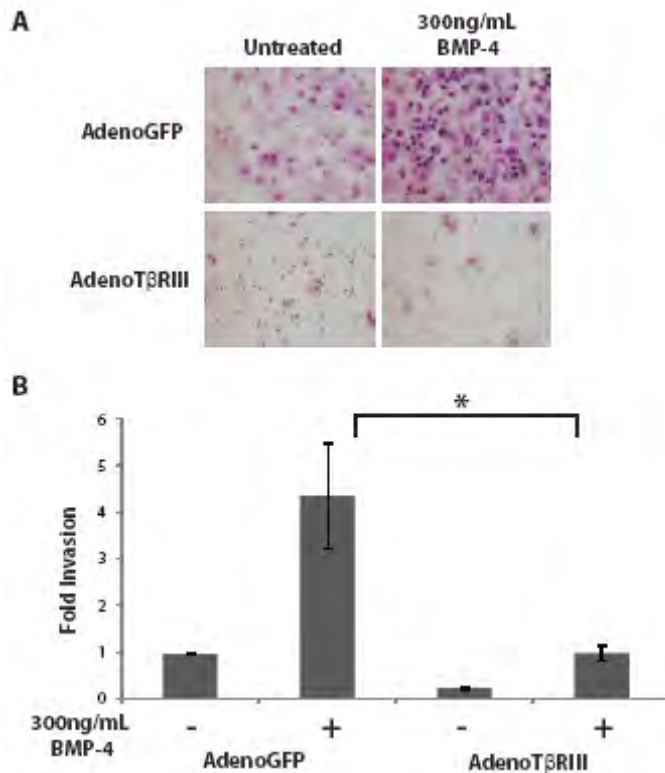


Figure 4. T β RIII decreases pancreatic cancer cell invasion. Panc-1 cells were adenovirally infected with T β RIII, followed by BMP-4-induced EMT. The cells were then plated in Matrigel invasion chambers. **A.** Representative images of the filters are shown. **B.** Invasive cells were counted and are graphically displayed.

A number of studies by our lab have determined that restoration of T β RIII expression in cancer cells resulted in inhibition of TGF- β induced migration and invasion. Therefore, we wanted to determine the effect of restoring T β RIII expression on BMP-mediated increases in cancer cell invasion. Adenoviral expression of T β RIII dramatically inhibited BMP-mediated increases in invasiveness (**Figure 3**). These data indicate that an increase in BMP expression by cancer cells results in a decrease in T β RIII and a subsequent increase in cancer cell invasion. In addition, these data suggest that if cancer cells maintain expression of T β RIII, the invasiveness of these cancer cells dramatically inhibits both TGF- β and BMP-mediated invasion. We were also able to demonstrate that this increase in invasion is mediated by an increase in extracellular matrix degrading proteins, matrix metalloproteinase-2, is increased as a consequence of Smad1 signaling

(**data not shown**). These data suggest a potential therapeutic approach to prevent cancer

cell metastasis. These studies established the foundation for a dynamic interplay between T β RIII and BMP in cancer biology. Future studies in Dr. Gerard Blobe's Lab will use this information as the foundation to better understand breast cancer biology.

Conclusions:

During the course of my funding by this pre-doctoral training grant, I have uncovered a novel role for T β RIII. I have identified and characterized T β RIII as a BMP cell surface receptor. In addition, I was part of a team that identified that T β RIII is frequently lost during breast cancer progression. The loss of T β RIII increases the migration and invasion of breast cancer cells *in vitro* and metastasis of the 4T1 model of breast cancer cells *in vivo*. We have also demonstrated that T β RIII expression is decreased by BMP in cancer cells, a potential mechanism for loss of expression since several BMP ligands have been shown to be increased in expression by cancer cells. Restoring T β RIII inhibits this BMP-induced invasion, a potential cancer therapy.

These data expand our understanding of the function and contribution of T β RIII to cellular responses to extracellular ligands, specifically BMP family members. I believe that the data I have presented reveal that T β RIII plays a central role in mediating TGF- β superfamily responses depending on cell type, along with the abundance and relative expression of each of its ligands.

Future Directions:

The studies presented here have examined and expanded our understanding of the contribution of T β RIII to BMP signaling and cellular responses. Future studies will be aimed at determining the hierarchy of ligand binding to T β RIII to better understand how cells determine a particular cellular response. These data have only begun to elucidate the role of T β RIII in BMP signaling. Future studies will be aimed at using the molecular data and cellular responses in the cancer cell types presented here to identify the function and contribution of T β RIII and its association with BMP family members, along with the BMP type I receptors (ALK3 and ALK6) in breast cancer. We understand that there is still quite a bit of information regarding T β RIII in breast cancer that remains to be determined and more work that needs to be done.

Through this training grant, I have been a integral part of determining the role of T β RIII in breast cancer. Our lab has demonstrated that maintained expression of T β RIII in a number of cancer cell types, including breast cancer, inhibits migration, invasion and metastasis of breast cancer cells. However, the mechanism remains to be elucidated, which we believe we have begun to partially uncover in studies performed in pancreatic cancer cells, which can be applied to breast cancer. Future studies will examine whether BMP family members are also able to induce EMT in other cell types, including breast cancer cell models, and whether this leads to an increase in the migration, invasion and the correlated to metastasis of breast cancer cells. We would also like to examine whether the decrease in lung metastases of the 4T1 mouse model of breast cancer is a consequence of alterations in BMP-mediated signaling [4]. The studies presented here laid the foundation to develop future studies aimed at answering whether BMP-mediated loss of T β RIII and increases in invasion increase breast cancer metastasis to the bone.

Key Research/Training Accomplishments:

Over the past year, I have added the following data to our understanding of the role of T β RIII in BMP-mediated signaling and carcinogenesis:

- Loss of T β RIII alters cellular responses to BMP. In PC-3 (prostate cancer cells), loss of T β RIII decreased cell sensitivity to BMP-2, while loss of T β RIII in Panc-1 (pancreatic cancer cells) increased cell sensitivity to BMP-2 and had no effect on MDA-MB-231 (breast cancer cells) as assayed by Smad1 phosphorylation (**Statement of Work (SOW) Task 2Ai; Appendix A, Figure 6**).
- T β RIII is required for BMP-2 mediated endothelial to mesenchymal transition (EndMT) during avian heart development (**SOW Task 2 and Task 3; Appendix A, Figure 7**).
- T β RIII mediates the ability of ALK6 to interact with β -arr2 (**SOW Task 2B, Figure 1**).
- T β RIII and β -arr2 together increase transcriptional responses downstream of ALK-6-mediated signaling (**SOW, Task 2A and 2C; Figure 2**).
- BMP family members induce epithelial to mesenchymal transition (EMT) (**SOW Task 3; data not shown**).
- BMP-induced EMT downregulates T β RIII expression (**SOW Task 1 and Task 3; data not shown**).
- Restored expression of T β RIII inhibits cancer cell invasion mediated by both TGF- β and BMP (**SOW Task 3Ai; Figure 3**).
- Smad1 activation is important for BMP-induced increases in pancreatic cancer cell invasiveness and T β RIII inhibits Smad1 phosphorylation in these cells (**SOW Task 3, data not shown**).

Reportable outcomes since last annual report:**A. Academic Training Accomplishments**

- Defended Doctoral thesis and received PhD in Molecular Cancer Biology.

B. Papers (published)

1. **Kirkbride KC**, Townsend TA, Bruinsma MW, Barnett JV and Blobe GC. *Bone Morphogenetic Proteins signal through the Transforming Growth Factor- β Type III Receptor*. **Journal of Biological Chemistry** (2008) March; **283** (12):7628-37.

C. Papers (Drafted)

1. Gordon KJ*, **Kirkbride KC*** and Blobe GC. *Bone Morphogenetic Proteins induce pancreatic cancer cell invasion through MMP-2*. (Submitted) (*contributed equally)
2. **Kirkbride KC***, Lee NY*, Sheu R and Blobe GC. *The Transforming Growth Factor- β Type III Receptor Mediates Differentially Internalization of the BMP Type I Receptors to Alter Signaling*. (Manuscript in preparation) (contributed equally)
3. **Gatza CE***, **Kirkbride KC***, Serwer LP, Sheu RD*, Dong MD*, You HJ*, How T*, Fields T and Blobe GC. *The Transforming Growth Factor- β Type III Receptor enhances colon cancer proliferation*. (Manuscript in preparation). (*contributed equally)

Summary of Breast Cancer Training and Accomplishments:**A. Academic Training Accomplishments**

- Completed course work and academic requirements for a Doctoral degree in Molecular Cancer Biology.
- Completed course work to receive a Certificate in Teaching College Biology. This training accomplishment will be used to facilitate communication and education of a broad audience on cancer biology. The skills I gained will be used in the future when teaching cancer biology lectures/courses.

B. Papers focused on breast cancer

1. Dong M, How T*, **Kirkbride KC***, Gordon KJ*, Lee JD*, Hempel N*, Kelly P, Moeller BJ, Marks J and Blobe GC. *The type III TGF- β Receptor suppresses breast cancer progression.* **Journal of Clinical Investigation** (2007) Jan; **117**(1):206-217. (*contributed equally)

C. Papers focused on the molecular mechanisms of T β RIII in BMP signaling

1. **Kirkbride KC**, Townsend TA, Bruinsma MW, Barnett JV and Blobe GC. *Bone Morphogenetic Proteins signal through the Transforming Growth Factor- β Type III Receptor.* **Journal of Biological Chemistry** (2008) March; **283** (12):7628-37.
2. Gordon KJ*, **Kirkbride KC*** and Blobe GC. *Bone Morphogenetic Proteins induce pancreatic cancer cell invasion through MMP-2.* (Submitted) (*contributed equally)

D. Breast cancer career training

- Developed molecular biology skills that will be essential for future cancer research.
- Established working relationships with other breast cancer research scientists.
- Established myself in the breast cancer research field through publication in peer-reviewed journals, along with oral presentation of research data at local and national meetings.
- Was an integral participant in animal modeling of breast cancer research using 4T1 cells to better understand the role of T β RIII in primary breast cancer tumor growth, along with metastasis. A tool I can apply to future breast cancer studies.

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Appendices:

- A. **Kirkbride KC**, Townsend TA, Bruinsma MW, Barnett JV and Blobe GC. *Bone Morphogenetic Proteins signal through the Transforming Growth Factor- β Type III Receptor*. **Journal of Biological Chemistry** (2008) March; **283** (12):7628-37.
- B. Current CV

Bone Morphogenetic Proteins Signal through the Transforming Growth Factor- β Type III Receptor^{*S}

Received for publication, June 13, 2007, and in revised form, January 9, 2008 Published, JBC Papers in Press, January 9, 2008, DOI 10.1074/jbc.M704883200

Kellye C. Kirkbride[‡], Todd A. Townsend[§], Monique W. Bruinsma[¶], Joey V. Barnett[§], and Gerard C. Blobe^{‡¶1}

From the Departments of [‡]Pharmacology and Cancer Biology and [¶]Medicine, Duke University, Durham, North Carolina 27708 and the [§]Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

The bone morphogenetic protein (BMP) family, the largest subfamily of the structurally conserved transforming growth factor- β (TGF- β) superfamily of growth factors, are multifunctional regulators of development, proliferation, and differentiation. The TGF- β type III receptor (T β RIII or betaglycan) is an abundant cell surface proteoglycan that has been well characterized as a TGF- β and inhibin receptor. Here we demonstrate that T β RIII functions as a BMP cell surface receptor. T β RIII directly and specifically binds to multiple members of the BMP subfamily, including BMP-2, BMP-4, BMP-7, and GDF-5, with similar kinetics and ligand binding domains as previously identified for TGF- β . T β RIII also enhances ligand binding to the BMP type I receptors, whereas short hairpin RNA-mediated silencing of endogenous T β RIII attenuates BMP-mediated Smad1 phosphorylation. Using a biologically relevant model for T β RIII function, we demonstrate that BMP-2 specifically stimulates T β RIII-mediated epithelial to mesenchymal cell transformation. The ability of T β RIII to serve as a cell surface receptor and mediate BMP, inhibin, and TGF- β signaling suggests a broader role for T β RIII in orchestrating TGF- β superfamily signaling.

Members of the transforming growth factor- β (TGF- β)² superfamily (including the TGF- β , the activin/inhibin, and the bone morphogenetic protein (BMP)/growth differentiation factor (GDF) subfamilies) are involved in many cellular processes including growth regulation, migration, apoptosis, and differentiation (1–4). The BMP subfamily, with 20 members, is the largest and has essential roles in development and well established roles in bone formation (1, 5).

BMP initiates signaling upon ligand binding to the high affinity type I BMP signaling receptors, activin-like receptor

kinase-1 (ALK1) (6), ALK2, ALK3, or ALK6 (7). The serine/threonine kinase activity of the type I receptor is activated upon recruitment and phosphorylation by a type II receptor, either the BMP type II receptor (BMPRII), or one of the activin type II receptors (ActRII or ActRIIB) (8). Upon activation the type I receptor phosphorylates the intracellular effector proteins, Smad1/5/8 transcription factors, which complex with the common Smad, Smad4, and enter the nucleus to induce BMP-mediated target gene transcription (1). Whereas most BMPs are able to elicit distinct cellular effects, the mechanism by which a limited number of cell surface receptors mediate these divergent effects remains to be established.

Co-receptors are important components of many signaling pathways (9). The TGF- β type III receptor (T β RIII or betaglycan), endoglin (10), and members of the repulsive guidance molecule family, DRAGON, RGMA, and hemojuvelin (11–13), have been characterized as TGF- β superfamily co-receptors. T β RIII is an abundantly and ubiquitously expressed cell surface receptor that enhances binding of all three isoforms of TGF- β to the TGF- β signaling receptor complex (14), and is required for high affinity cell surface binding of TGF- β 2. T β RIII also binds inhibin, another TGF- β superfamily member (15). In addition to directly regulating ligand availability, T β RIII also alters the subcellular localization of the signaling receptor complex through interactions with the PDZ domain containing protein, GIPC (16), and β -arrestin2 (17). The demonstration that T β RIII is required for TGF- β 2-stimulated epithelial to mesenchymal transformation (EMT) *in vitro* (18) and the embryonic lethality of the T β RIII knock-out mouse (19, 20) has fostered consideration of a unique and non-redundant role for T β RIII that is independent of ligand presentation to the kinase receptor complexes.

Several observations suggest that T β RIII may serve as a cell surface receptor for BMP. First, BMP shares structural similarity with ligands known to bind T β RIII (21). Second, T β RIII shares extracellular domain homology with endoglin (22, 23), which binds BMP-2 and BMP-7 in the presence of their respective type II receptors (24). Finally, T β RIII is a heparan sulfate proteoglycan (25, 26) and these glycosaminoglycan modifications have been shown to mediate basic fibroblast growth factor binding to T β RIII (27). As BMP has a strong affinity for heparan sulfate (28), these modifications may confer the ability of T β RIII to bind BMP as well. Here we investigate whether T β RIII functions as a cell surface receptor for BMP.

^{*} This work was supported by a predoctoral fellowship from the Department of Defense Breast Cancer Research Program (to K. C. K.), Vanderbilt University Grant GM007628 (to T. A. T.), and National Institutes of Health Grants HL52922 (to J. V. B.) and CA106307 (to G. C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1–S4 and Figs. S1–S3.

¹ To whom correspondence should be addressed: 354 LSRC Research Dr., Box 91004 DUMC, Durham, NC 27708. Tel.: 919-668-1352; Fax: 919-681-6906; E-mail: blobe001@mc.duke.edu.

² The abbreviations used are: TGF- β , transforming growth factor- β ; ALK, activin receptor-like kinase; AV, atrioventricular; BMP, bone morphogenetic protein; EMT, epithelial to mesenchymal transition; GDF, growth/differentiation factor; T β RIII, TGF- β type III receptor; sT β RIII, soluble T β RIII; T β RIII Δ gag, T β RIII minus glycosaminoglycan chains; shRNA, short hairpin RNA; GFP, green fluorescent protein.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, Antibodies, and Growth Factors—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Invitrogen). NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. PC-3 cells were maintained in F12 Kaighn's (Invitrogen) supplemented with 10% fetal bovine serum.

Human TβRIIIΔgag was generated using XL-Site directed mutagenesis (Stratagene) to mutate serine 532 to alanine (forward: 5'-CCCTTGGGGACAGTGCTGGTTGGCCAGA and reverse 5'-CATCTGGCCAACCAGCACTGTCCCCAAG), followed by mutating serine 543 to alanine (forward: 5'-GGA-AATCCATTATCACCTGCCTCCAGAT and reverse 5'-GGT-TATGAAGATCTGGAGGCAGGTGATA) to make the double mutant. Plasmids were generous gifts from Kohei Miyazono (ALK3 and ALK6), Petra Knaus (BMPRII), and Fernando Lopez-Casillas (myc-TβRIII extracellular domain deletions) (29).

Adenoviruses for EMT assays were generated using the pAdEasy system (30). All concentrated viruses were titered by performing serial dilutions of the concentrated virus and counting the number of GFP-expressing 293 cells after 18–24 h.

Adenoviruses containing sequences for human TβRIII and non-targeting control short hairpin RNA were generated by Dharmacon and inserted into a vector co-expressing the DS-Red fluorophore using the Adeno-X™ Expression System (Clontech). Specificity for TβRIII has previously been demonstrated (31, 32). Recombinant human BMP-2, BMP-4, BMP-7, GDF-5, and soluble TβRIII were purchased from R&D Systems.

BMP receptors were detected with an anti-hemagglutinin antibody (Roche). A polyclonal antibody against the cytoplasmic tail of TβRIII was generated by our laboratory and characterized previously (33). The β-actin antibody was purchased from (Sigma). Both anti-mouse and anti-rabbit antibodies were purchased from Amersham Biosciences.

Iodination of BMP Family Members—¹²⁵I-BMP were generated using the chloramine T method as previously described (34). Ten micrograms of carrier-free recombinant human BMP-2, BMP-7, BMP-4, and GDF-5 were used for each labeling. ¹²⁵I-TGF-β1 was purchased from Amersham Biosciences.

Cross-linking and Immunoprecipitation of Receptors—Binding assays were performed as previously described (35). COS-7 cells (150,000 cells/well in 6-well plates) were transiently transfected with 2 μg (or otherwise indicated) of plasmid DNA using FuGENE 6 (Roche) 18 h after plating. Transiently transfected cells were incubated for 3 h at 4 °C with 10 nM BMP (150 pM TGF-β1), unless indicated otherwise. Endogenous TβRIII studies were incubated overnight at 4 °C. Competition studies were carried out similarly, except that the indicated concentrations of cold BMP-7 (×0.1, 1, 10, and 100) were added alongside the hot ligand (2 nM) for 3 h at 4 °C. Cell surface complexes were cross-linked with disuccinimidyl suberate and quenched with 1 M glycine. The cells were then lysed with RIPA buffer containing protease inhibitors and immunoprecipitated at 4 °C. Before immunoprecipitation lysate was removed for control gel anal-

ysis. The immunoprecipitated proteins were resolved using SDS-PAGE. These gels were subsequently dried and exposed to an autoradiograph.

Surface Plasmon Resonance—BMP-2 (1600 response units) was immobilized on a CM5 sensor chip using amine coupling (sodium acetate pH 4.5). Soluble TβRIII (R & D Systems) was diluted in running buffer (10 mM Hepes, pH 7, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20) and flowed at concentrations ranging from 7.81 to 250 nM for 5 min at a flow rate of 50 μl/min. TGF-β1 (900 response units) was immobilized in sodium acetate, pH 5.5. In the TGF-β studies, sTβRIII (concentrations ranging from 8.75 to 560 nM) was flowed at a rate of 50 μl/min for 3 min. The resulting sensograms were then fit using nonlinear least squares analysis and numerical integration of differential rate equations and the fits were then analyzed by considering the distribution of the residuals.

Smad1 Phosphorylation—PC-3 cells were plated at 125,000 cells/well and infected with adenovirus (50 multiplicity of infection) containing either non-targeting control short hairpin RNA (shRNA) or shRNA directed against human TβRIII 24 h after plating. The cells were then incubated for 96 h, serum-starved for 5 h, and treated with the indicated concentrations of rhBMP-2 for 10 min followed by direct lysis. Smad1 phosphorylation was assayed by Western blot with phospho-Smad1 antibody (Cell Signaling), with total Smad1 antibody as a loading control (Cell Signaling).

Viral Injections and Collagen Gel Assays—Injections and assays were performed as previously described by Desgrosellier *et al.* (36) with the exception of the addition of vehicle (bovine serum albumin/HCl), 200 pM TGF-β2, or 5 nM BMP-2, BMP-4, BMP-7, or GDF-5 12 h after placement of the explant on collagen. Each GFP-expressing cell was scored as epithelial, activated, or transformed as described (36). For the total number of explants and cells counted, refer to supplemental Tables 3 and 4.

RESULTS

TβRIII Is a Cell Surface Receptor for BMP-2—To determine whether TβRIII functions as a BMP receptor, we expressed TβRIII, along with the BMP receptors, ALK3, ALK6, or BMPRII, in COS-7 cells, which express low endogenous levels of these cell surface receptors and assessed BMP-2 binding by chemically cross-linking ¹²⁵I-BMP-2 to binding partners on the cell surface. As expected, ¹²⁵I-BMP-2 bound to ALK3 and ALK6, but not to BMPRII, which cannot bind ligand on its own (Fig. 1A). ¹²⁵I-BMP-2 was also detected bound to TβRIII in the presence of ALK3, ALK6, and BMPRII (Fig. 1A, lanes 3, 5, and 7) suggesting that TβRIII binds BMP-2. TβRIII expression also modestly increased BMP-2 binding to ALK3 and ALK6, but did not confer ligand binding to BMPRII (Fig. 1A).

BMPRII binds BMP ligands only when in complex with either ALK3 or ALK6 (8). To determine whether TβRIII affects the ability of BMPRII to bind BMP in the presence of ALK3 or ALK6, we expressed TβRIII with these traditional BMP signaling complexes. As expected, BMPRII could be detected bound to ¹²⁵I-BMP-2 when co-expressed with ALK3 or ALK6 (Fig. 1B, lanes 1 and 3), and TβRIII did not significantly alter BMP-2 binding to BMPRII (Fig. 1B, lanes 2 and 4).

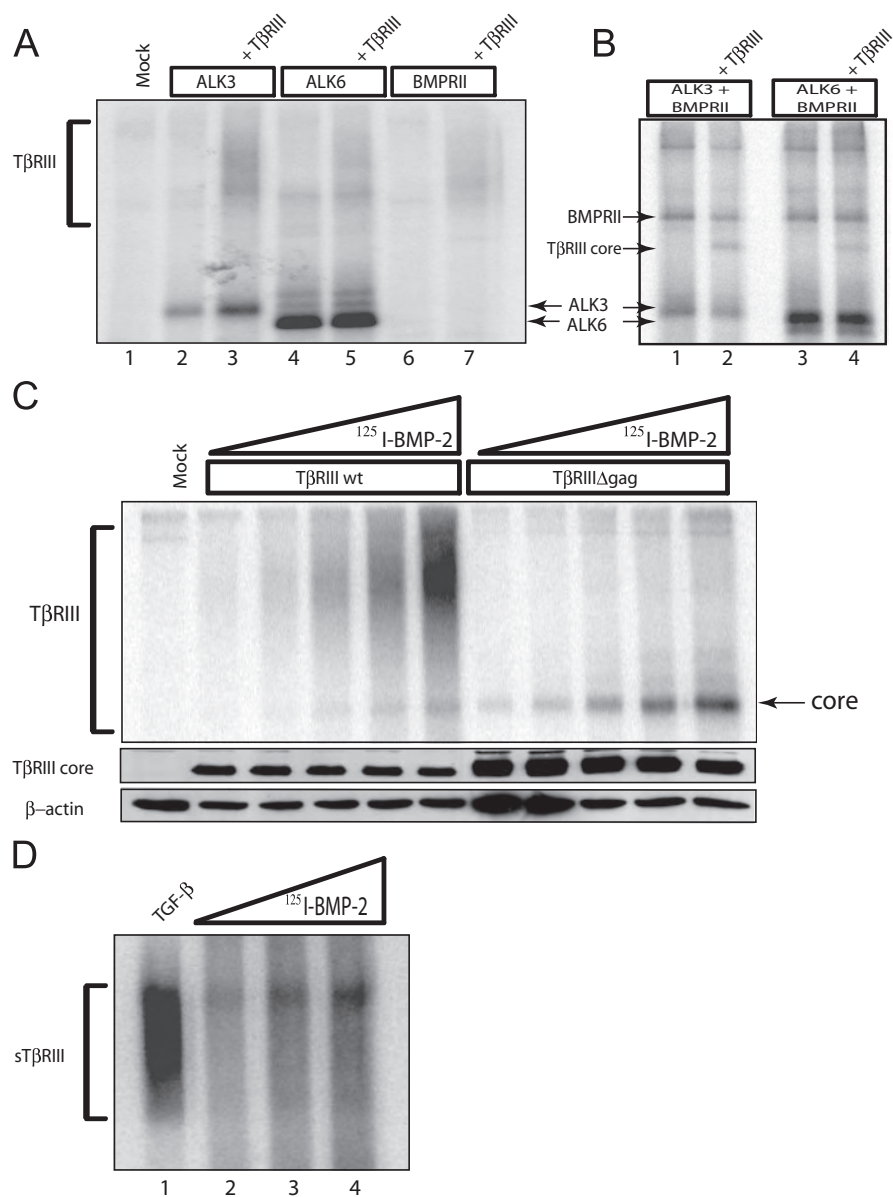


FIGURE 1. BMP-2 binds to the extracellular domain of TβRIII. COS-7 cells expressing hemagglutinin-tagged ALK3, ALK6, or BMPRII (A); ALK3 or ALK6 and BMPRII (B) with or without wild-type (wt) human TβRIII were exposed to 10 nM ¹²⁵I-BMP-2, cross-linked, immunoprecipitated, and separated by SDS-PAGE and detected by phosphorimaging. C, COS-7 cells expressing wt TβRIII or TβRIII with serine to alanine mutations at glycosaminoglycan attachment sites (TβRIIIΔgag) were exposed to increasing doses of ¹²⁵I-BMP-2 (2, 5, 10, 25, and 50 nM, respectively) (top panel). Total cellular TβRIII and β-actin are shown as expression and loading controls (bottom panels). D, soluble TβRIII (sTβRIII) was exposed to ¹²⁵I-TGF-β1 (150 pM) as a positive control and increasing doses of ¹²⁵I-BMP-2 (2, 5, and 10 nM) followed by chemical cross-linking and immunoprecipitation with an antibody against the extracellular domain of TβRIII. The proteins were then resolved by SDS-PAGE. The data are representative of three independent experiments.

To determine whether BMP receptor expression was necessary for the ability of TβRIII to bind BMP-2, we expressed TβRIII alone. In the absence of ALK3 and ALK6, ¹²⁵I-BMP-2 formed a cross-linked complex with fully processed TβRIII in a dose-dependent fashion (Fig. 1C, left) establishing that BMP-2 is able to bind TβRIII independent of other ligand binding receptors.

BMPs bind heparan sulfate with high affinity (28, 37) and TβRIII is a heparan sulfate and chondroitin sulfate proteoglycan (38). These glycosaminoglycan modifications are important for basic fibroblast growth factor binding to TβRIII (27),

but not for TGF-β (38) or inhibin binding (39). To determine whether these glycosaminoglycan modifications were important for BMP binding to TβRIII, we used a mutant of TβRIII in which the serines (Ser-535 and Ser-546) necessary for glycosaminoglycan chain attachment are converted to alanines preventing this modification (TβRIIIΔgag) (38). In these studies, the core protein of TβRIIIΔgag was affinity labeled with ¹²⁵I-BMP-2 in a dose-dependent fashion (Fig. 1C, right) indicating that the heparan sulfate modifications were not necessary for BMP-2 binding to TβRIII.

TβRIII exists in two forms, a membrane bound form and a soluble form, sTβRIII, derived from ectodomain shedding (38). sTβRIII consists of the extracellular domain of TβRIII and is able to bind TGF-β, sequester ligand from the cell surface receptors, and antagonize TGF-β signaling (38, 40). To determine whether sTβRIII is able to bind BMP-2, we exposed recombinant, purified sTβRIII to ¹²⁵I-BMP-2. As with membrane-bound TβRIII, sTβRIII was affinity labeled with ¹²⁵I-BMP-2 in a dose-dependent fashion (Fig. 1D, lanes 2–4), with a BMP-2 binding pattern similar to that of the well characterized TβRIII ligand, TGF-β1 (Fig. 1D, lane 1). These data demonstrate that sTβRIII is able to bind BMP-2 and confirm that the binding of BMP-2 to the extracellular domain of TβRIII is direct.

Kinetics and Affinity of BMP Binding to TβRIII—To characterize the interaction between TβRIII and BMP-2 we used surface plasmon resonance (also known as BIAcore), a sensitive method to measure protein-protein interactions (41, 42). BIAcore has been used to define the interactions of multiple TGF-β superfamily ligands with their receptors (6, 43–45). Both BMP-2 and TGF-β1 were immobilized to a dextran sensor chip and purified sTβRIII was the analyte. Upon mathematically fitting the response curves, the model that best fit the binding of TβRIII to BMP-2 was the bivalent analyte (or avidity) model (Fig. 2 and supplemental Table S1). This model also provided the best fit for TGF-β1 binding to TβRIII, based on previous BIAcore studies (44) and confirmed here (supplemental Fig. 1). The fit to the bivalent analyte model suggested two ligand binding sites on TβRIII for

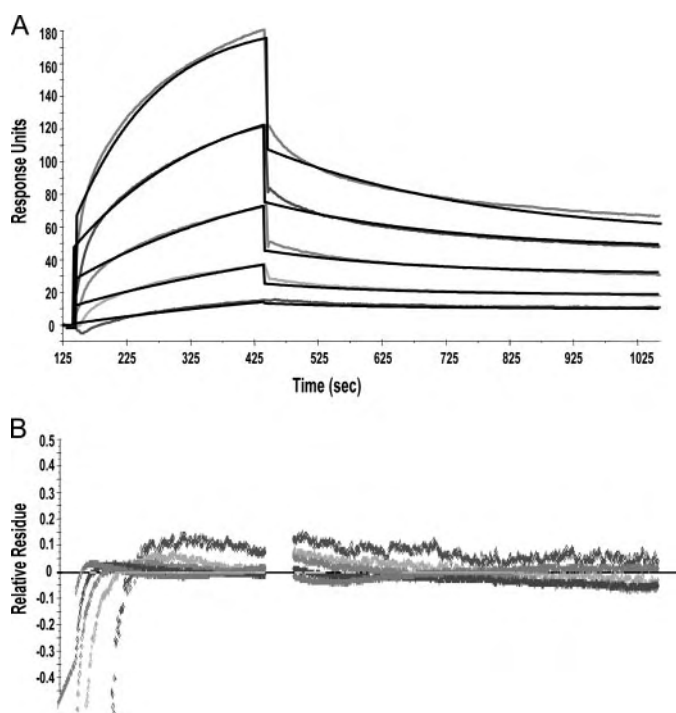


FIGURE 2. The bivalent analyte model best fits the kinetics for BMP-2 binding to TβRIII using surface plasmon resonance (BIAcore). A, global fitting analysis was carried out on the response units of TβRIII (the analyte) binding to rhBMP-2 (1600 response units) immobilized using amine coupling to a CM5 dextran sensor chip after subtracting out bovine serum albumin binding as background. TβRIII was flowed over BMP-2 at a rate of 50 ml/min for 5 min at concentrations ranging from 7.81 to 250 nM. The data were then fit to kinetic models. Represented is the best fit of the data, the bivalent analyte (also known as the avidity model). This data are representative of three independent experiments. B, graphical representation of the residuals of the data fit to the bivalent analyte model.

both TGF-β1 and BMP-2 (supplemental Table S2). Using the bivalent model, we established kinetic and thermodynamic constants for BMP-2 interacting with the ligand binding site on sTβRIII, with data from three independent experiments establishing a dissociation constant of 10 μM (±3.66) for BMP-2 and 5 μM (±1.71) for TGF-β1. These results are comparable with previously published reports for TβRIII and TGF-β1 (44). These studies indicate that the affinity of TβRIII for BMP-2 is on the same order of magnitude as the affinity of TβRIII for TGF-β1.

Multiple Members of the BMP Subfamily Bind to TβRIII—To determine whether TβRIII could bind other BMP subfamily members, we radiolabeled representative members of the three distinct BMP subfamilies, BMP-4, BMP-7, and GDF-5 (Fig. 3A) (1). ¹²⁵I-BMP-2, ¹²⁵I-BMP-4, ¹²⁵I-BMP-7, and ¹²⁵I-GDF-5 each formed a cross-linked complex with both the fully processed form of TβRIII, along with TβRIIIΔgag (Fig. 3B), suggesting that a broad range of BMP family members can bind to the core protein of TβRIII. Intriguingly, there were subtle differences in the binding patterns of certain BMP subfamily members to TβRIII, particularly to the core protein.

To define the specificity of the interaction of BMP with TβRIII, we performed competition experiments with iodinated ligand in the presence of increasing concentrations of unlabeled ligand. Consistent with a previous report (46), we were unable to specifically compete off BMP-2 binding with excess cold

ligand (data not shown). The nonspecific binding of ¹²⁵I-BMP-2 is likely due to the presence of basic residues at the amino terminus of BMP-2, as previously reported (46). Accordingly, we investigated specificity using BMP-7, which also binds TβRIII, and for which these amino-terminal basic residues are not present. Here, 100-fold excess cold ligand successfully competed off ¹²⁵I-BMP-7 from wild-type TβRIII (Fig. 3C) with ~40% nonspecific binding remaining (Fig. 3C and supplemental Fig. S2). Unlabeled BMP-7 also competed with ¹²⁵I-BMP-7 for binding to TβRIIIΔgag (Fig. 3D). Taken together, these data support the ability of TβRIII to specifically bind a broad range of BMP subfamily members.

To establish the physiological relevance of BMP binding to TβRIII, we investigated whether BMP family members bound to endogenous TβRIII. For these studies, we used NIH3T3 cells, which abundantly express TβRIII. Both ¹²⁵I-BMP-2 and ¹²⁵I-BMP-7 bound to high molecular weight complexes corresponding to the fully processed endogenous form of TβRIII, which were specifically immunoprecipitated by a TβRIII antibody, and not by preimmune serum, in a pattern similar to that of ¹²⁵I-TGF-β1 (Fig. 3E). These data confirm that both BMP-2 and BMP-7 are able to bind to endogenous TβRIII.

BMP Binds to Both TβRIII Ligand Binding Domains—Our BIAcore data suggested two ligand binding sites for BMP-2 and TGF-β1 on the core protein of TβRIII. Consistent with this, previous studies have established two TGF-β binding regions on TβRIII, with one in the membrane-distal half (Binding Region 1) and one in the membrane-proximal half (Binding Region 2) (Fig. 4A) (29, 47). In contrast to TGF-β, inhibin binds selectively to Binding Region 2 (39). To further investigate BMP binding to TβRIII, we defined the regions of TβRIII that mediate BMP binding. We expressed extracellular domain deletions of TβRIII, including those lacking either Binding Regions 1 or 2 and then assessed their ability to bind BMP-2 and BMP-7. Both ¹²⁵I-BMP-2 (Fig. 4B) and ¹²⁵I-BMP-7 (Fig. 4C) exhibited a binding pattern identical to that of ¹²⁵I-TGF-β1 (Fig. 4D). BMP-2 and BMP-7 bound TβRIII mutants with either Binding Region 2 (Fig. 4, B and C, lanes 4 and 5) or Binding Region 1 (Fig. 4, B and C, lane 6) deleted. In contrast, when portions of both of these regions are deleted, no binding occurred for either BMP-2 or BMP-7 (Fig. 4, B and C, lane 3), providing further support for specific binding of both BMP-2 and BMP-7 to the other TβRIII constructs. In addition, like TGF-β1, BMP-2 and BMP-7 appear to preferentially bind Binding Region 1 (Fig. 4, B and C, compare lanes 5 and 6), which is the region most similar to endoglin. These data establish that both BMP-2 and BMP-7 can bind to either of the two ligand binding motifs of TβRIII, similar to TGF-β1, validating the bivalent model for BMP binding to TβRIII.

TβRIII Enhances Ligand Binding to ALK-3 and ALK-6—As a co-receptor, TβRIII has an established role in presenting ligand, leading to enhanced TGF-β binding to TβRII and increasing TGF-β signaling (35), while also enhancing inhibin binding to ActRII to facilitate inhibin-mediated antagonism of activin signaling (15). We observed a slight increase in BMP-2 binding to ALK3 and ALK6 in the presence of TβRIII (Fig. 1A). To determine whether TβRIII alters BMP binding to the BMP signaling receptors, the effect of increasing TβRIII expression

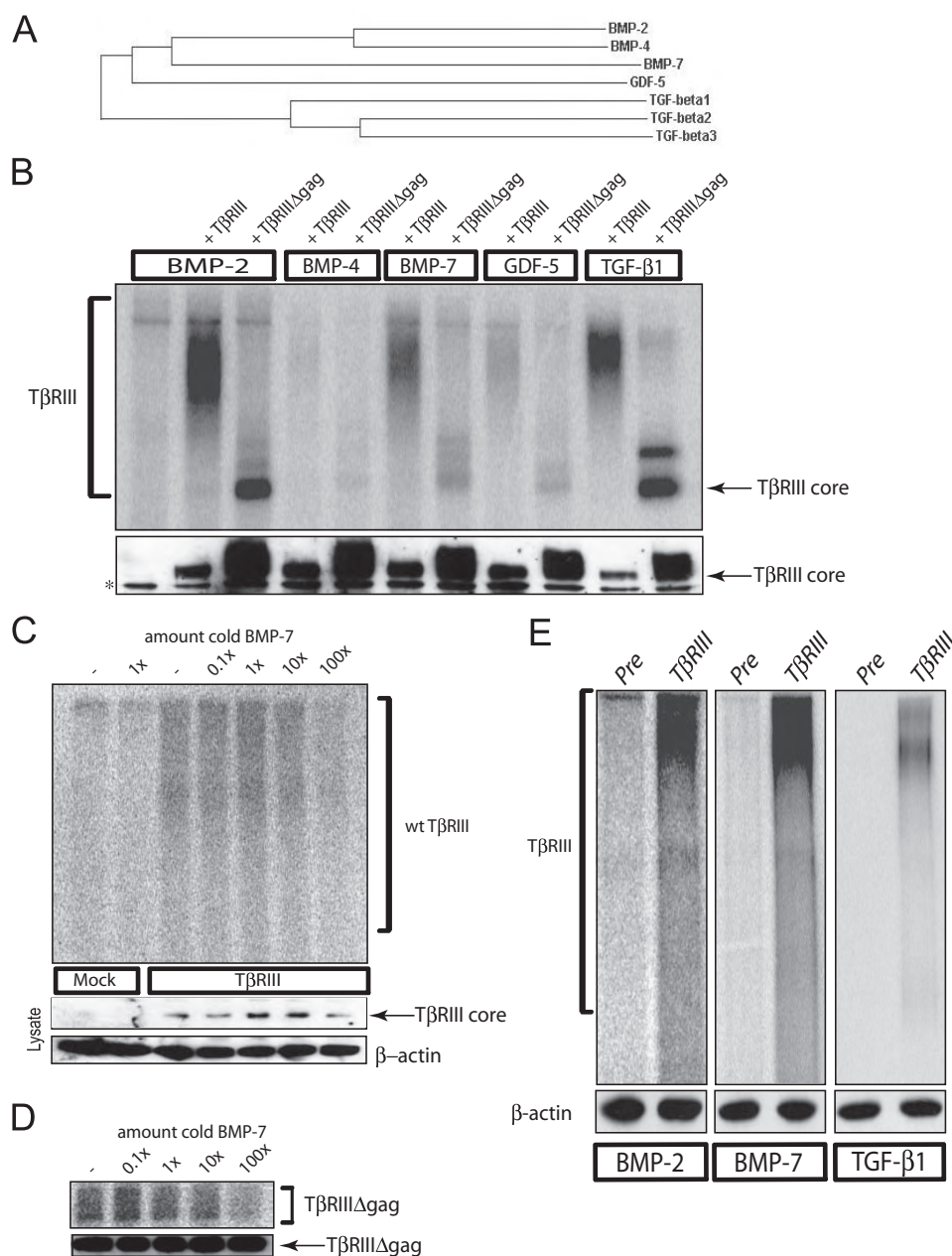


FIGURE 3. Multiple members of the BMP family specifically bind to the core protein of TβRIII. A, evolutionary tree diagram generated by the MacVector program from NCBI sequence alignment of known TβRIII ligands and the BMP members used in this study. B, COS-7 cells expressing wild type (wt) TβRIII or TβRIIIΔgag were exposed to ¹²⁵I-BMP-2, ¹²⁵I-BMP-4, ¹²⁵I-BMP-7, ¹²⁵I-GDF-5, or ¹²⁵I-TGF-β1 as indicated and chemically cross-linked followed by immunoprecipitation with an antibody to the cytoplasmic tail of TβRIII. Total cellular TβRIII is shown as an expression control (bottom panel). *, indicates a nonspecific band. C and D, COS-7 cells expressing wild type TβRIII (C) or TβRIIIΔgag (D) were simultaneously exposed to 2 nM ¹²⁵I-BMP-7 in the presence of increasing amounts of cold BMP-7 (0.2, 2, 20, and 200 nM) as indicated, followed by chemical cross-linking and immunoprecipitation. E, NIH3T3 cells were exposed to ¹²⁵I-BMP-2, ¹²⁵I-BMP-7, and ¹²⁵I-TGF-β1. All lysates were immunoprecipitated with either preimmune serum or a TβRIII antibody, separated by SDS-PAGE, and detected by phosphorimaging. The data are representative of three independent experiments.

on the binding of either ¹²⁵I-BMP-2 or ¹²⁵I-BMP-7 to ALK3 and ALK6 was examined. TβRIII significantly increased binding of ¹²⁵I-BMP-2 to ALK-3 in an expression-dependent manner (Fig. 5A), with a maximal 2-fold increase (Fig. 5C). Expression of TβRIII also significantly enhanced ¹²⁵I-BMP-2 binding to ALK6 ~2-fold (Fig. 5B) and ¹²⁵I-BMP-7 (Fig. 5D) binding to ALK6 about 3-fold (Fig. 5E). These increases in binding were

due to increasing TβRIII expression and not due to altered ALK3 or ALK6 levels. TβRIII expression did not enhance BMP-4 binding to ALK6 (supplemental Fig. 3) nor BMP-2 binding to BMPRII in the absence of either ALK3 or ALK6 (Fig. 1A, lanes 6 and 7), suggesting that TβRIII does not function to confer BMP ligand binding to BMP receptors unable to bind BMP subfamily members independently. Taken together, these data suggest that one function for TβRIII in binding BMP subfamily members is to enhance BMP binding to their respective ligand binding receptors, without altering ligand binding specificity.

Loss of Endogenous TβRIII Expression Reduces BMP Responsiveness—TβRIII-mediated ligand presentation to the signaling receptors increases signaling by the respective ligand, as has been demonstrated for TGF-β (35) and inhibin (15). To assess whether TβRIII-mediated BMP presentation to BMP receptors regulated BMP signaling, we used shRNA to decrease endogenous TβRIII expression and assessed effects on BMP-mediated Smad1 phosphorylation. As a model system we used the human prostate cancer cell line, PC-3, which express moderate levels of endogenous TβRIII (data not shown), express BMPRII, ALK3, and ALK6, and are BMP responsive, including BMP-induced Smad1 phosphorylation (48). PC-3 cells were infected with non-targeting control shRNA and human TβRIII shRNA, which concurrently expresses DS-Red fluorophore. Fluorescent images demonstrated similar infection efficiency and phase-contrast images demonstrated similar viability (data not shown). shRNA to TβRIII consistently decreased endogenous

TβRIII expression by greater than 50%, whereas the non-targeting control shRNA had no effect on endogenous TβRIII expression, as previously reported (31, 32) (data not shown). This loss of TβRIII expression was associated with a significant decrease in BMP-2-mediated Smad1 phosphorylation, particularly at low BMP-2 concentrations, where phospho-Smad1 induction was approximately 50% of that in non-tar-

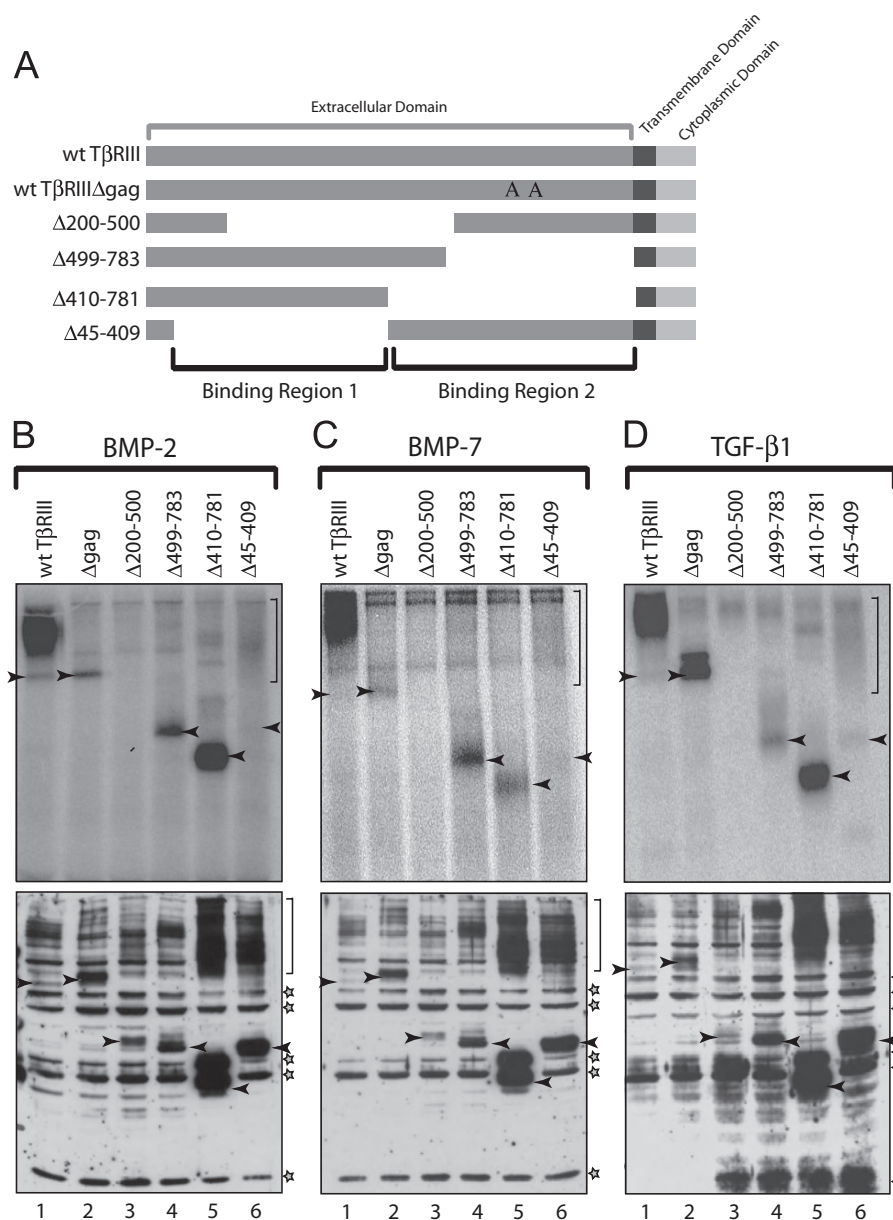


FIGURE 4. BMP binds to both TβRIII ligand and binding domains. A, diagram of deletions in the extracellular domain mutants of TβRIII (adapted from Ref. 38). B, autoradiograph (top) of COS-7 cells expressing myc-tagged extracellular domain deletions of TβRIII were treated with ^{125}I -BMP-2 (10 nM) (B), ^{125}I -BMP-7 (10 nM) (C), and ^{125}I -TGF-β1 (100 pM) (D) as indicated and chemically cross-linked followed by immunoprecipitation with an antibody to the cytoplasmic tail of TβRIII. Total cellular expression of the respective TβRIII mutants is shown as an expression control (bottom panel). Arrowheads indicate the core protein of the TβRIII mutants, brackets identify processed forms of TβRIII, and stars indicate major nonspecific bands. The data are representative of three independent experiments.

geting control cells (Fig. 6, A and B). These data suggest that TβRIII serves to present BMP-2 to either ALK3 or ALK6 and increase cellular sensitivity to BMP-2.

BMP-2-specific Interaction with TβRIII Yields a Functional Response—To establish further support for a role for TβRIII in BMP signaling, we used a well characterized model of TβRIII signaling that scores cell invasion into a three-dimensional collagen matrix to determine whether BMP-2 binding to TβRIII initiated signaling (49). During embryonic development, a subset of endothelial cells in the atrioventricular (AV) cushion of the heart undergo EMT as an early step in valve formation. This process has been well studied using explants of the AV cushion that have been

placed onto collagen gels. Endothelial cells from this AV cushion form a compact epithelial sheet composed of rounded, tightly packed cells on the surface of the collagen, whereas transformed cells are identified morphologically and functionally as those that elongate and invade into the collagen matrix. Endothelial cells in these AV cushions express TβRIII, as well as TβRII and TβRI, along with the BMP receptors, BMPRII and ALK3, and undergo EMT in response to exogenous TGF-β (18, 50). In contrast to AV cushion endothelial cells, ventricular endothelial cells lack TβRIII and do not undergo EMT, even in response to an excess of TGF-β. However, expression of TβRIII in these ventricular endothelial cells results in TGF-β2-induced EMT, demonstrating a unique requirement for TβRIII and suggesting a non-canonical role for TβRIII in addition to ligand presentation (18).

This *in vitro* assay system, where EMT is dependent on the presence of TβRIII, is currently the only described assay for TβRIII signaling (18). To determine whether BMP-2 is also involved in TβRIII-mediated transformation, chick ventricular endothelial cells expressing either GFP or TβRIII and GFP were incubated with TGF-β2 or BMP-2. Neither ligand alone induced transformation of control infected cells (Fig. 7E). However, expression of TβRIII conferred BMP-2- and TGF-β2-induced EMT (Fig. 7E) as measured by a 2-fold increase in the percentage of transformed cells (cells elongating and invading the collagen gel (Fig. 7, C and D)), and a concomitant decrease in the percentage of epithelial cells (cells rounded and remaining on the surface of the gel (Fig. 7, A and B)).

Importantly, BMP-2 (5 nM) induced transformation to a similar extent as 200 pM TGF-β2, and both BMP-2- and TGF-β2-induced transformation were entirely dependent on TβRIII expression. These data demonstrate that BMP-2 requires TβRIII to mediate this biological response, consistent with TβRIII and BMP-2 functioning as a receptor-ligand pair. Because other BMP family members also bind to TβRIII, we investigated the ability of these BMP members (BMP-4, BMP-7, and GDF-5) to induce EMT in this model system. Surprisingly, only BMP-2 induced EMT, suggesting a specific functional role for BMP-2 binding to TβRIII in mediating EMT during heart development (Fig. 7F).

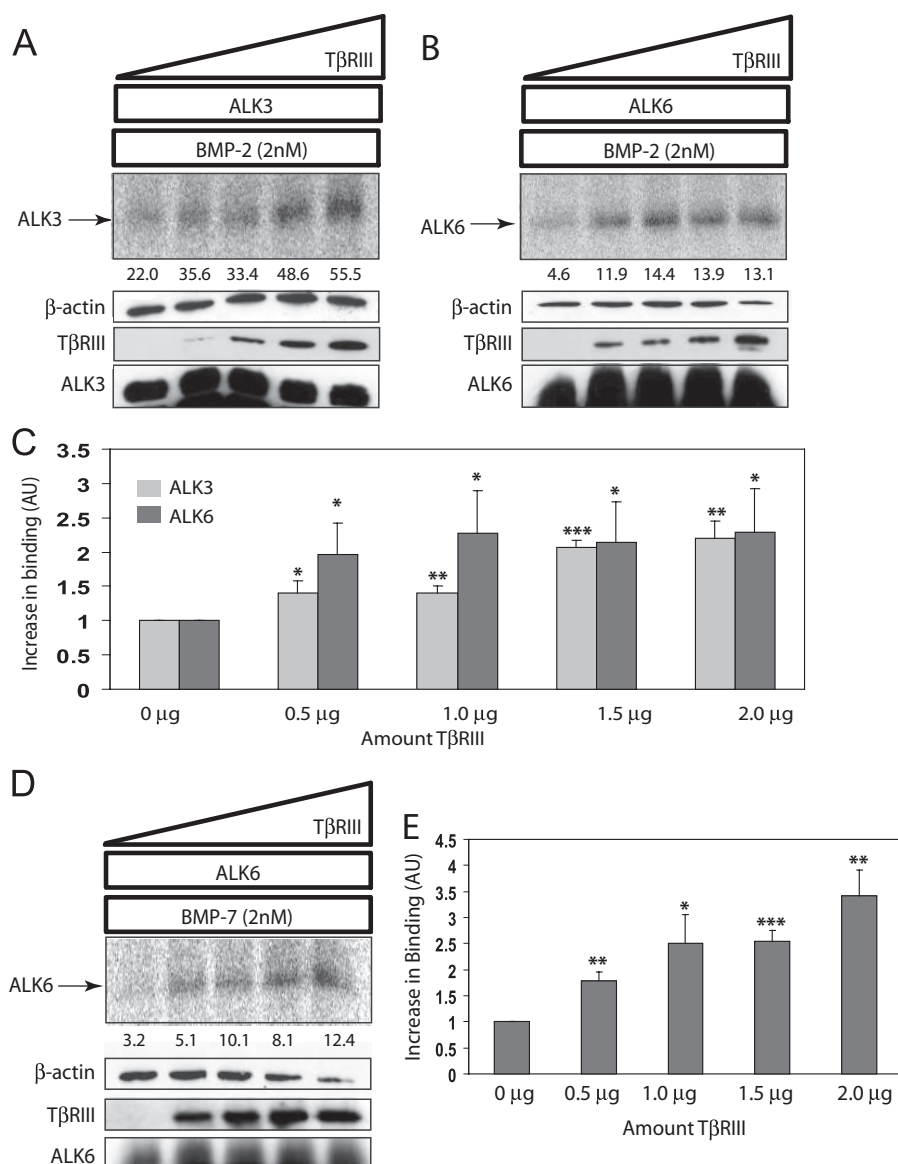


FIGURE 5. TβRIII presents BMP to the BMP type I receptors. COS-7 cells expressing either ALK3 (A) or ALK6 (B and D), in the absence or presence of increasing amounts of TβRIII (0, 0.5, 1.0, 1.5, and 2.0 μg, respectively), were exposed to 2 nM radiolabeled ligand (BMP-2 (A and B) or BMP-7 (D), as indicated) followed by chemical cross-linking and immunoprecipitation of the BMP receptor using an hemagglutinin antibody. The proteins were separated by SDS-PAGE and detected by phosphorimaging. β-Actin is shown as a loading control (bottom panel). Images are representative of three independent experiments. Graphical representation of the average change in signal intensity of BMP-2 binding to ALK3 and ALK6 (C) or BMP-7 binding to ALK6 (E) from three independent experiments. Densitometry using ImageJ software was used to determine the signal intensity. The densitometry of each band was normalized to the signal intensity of the ligand bound to the respective BMP receptor without TβRIII. Two-tailed Student's *t* test was used to determine statistical significance in comparison to the respective BMP receptor without TβRIII; *, *p* < 0.05; **, *p* < 0.005; ***, *p* < 0.0005.

DISCUSSION

Here we demonstrate, for the first time, that TβRIII is able to bind BMP, another class of ligands within the TGF-β superfamily. Importantly, TβRIII is able to bind a broad range of BMP ligands, including BMP-2, BMP-4, BMP-7, and GDF-5. We demonstrate that this binding is specific, through both competition studies and studies demonstrating that disruption of both ligand binding domains prevents BMP binding to TβRIII. Functionally, TβRIII serves as a BMP co-receptor by increasing BMP binding to the BMP signaling receptors, ALK3 and ALK6, and increasing BMP signaling, and as a BMP receptor in a bio-

logically relevant system. Whereas glycosylphosphatidylinositol-linked co-receptors have recently been identified for BMP (DRAGON, RGMa, and hemojuvelin) these receptors each have limited tissue distribution (12). In contrast, TβRIII is a ubiquitously expressed transmembrane receptor (51). TβRIII has previously been identified as a co-receptor for both TGF-β and inhibin based on its ability to bind these ligands and enhance their cellular effects. Data presented here suggest that TβRIII functions similarly for the BMP family of ligands. Given the broad tissue distribution of TβRIII and its ability to bind all major classes of ligands in the TGF-β superfamily, TβRIII is poised to act as a major orchestrator of TGF-β superfamily signaling. As members of the TGF-β superfamily are able to antagonize each other, including BMP-7 antagonism of TGF-β-induced fibrogenesis in mesengial cells (52) and distinct inhibin antagonism of activin and BMP signaling (53), TβRIII may be the common component for these TGF-β superfamily ligands mediating this antagonism. Consistent with this hypothesis, we have demonstrated that BMP-2 can compete with TGF-β1 for binding to TβRIII (data not shown). Current studies are delineating the hierarchy of ligand binding to TβRIII to further define the role of TβRIII as a moderator of TGF-β superfamily signaling.

Here we demonstrate that BMP-2 binding to TβRIII elicits a functional response in ventricular endothelium. These data are consistent with a suggested role for BMP-2 in normal cardiac development. A

recent report demonstrated that conditional ablation of BMP-2 in heart muscle, where it is expressed during development, results in failure of the TβRIII expressing endothelial cells in the adjacent valve forming region of the heart to undergo EMT (54, 55). Furthermore, we show that TβRIII has enhanced functional interaction with ALK3 in the presence of BMP-2. Conditional ablation of ALK3 from the endothelium also results in a failure of EMT in the valve forming regions (50). Taken together, these data suggest that BMP-2 produced by the myocardium may act directly through TβRIII on endothelial cells in the heart to stimulate EMT and valvulogenesis.

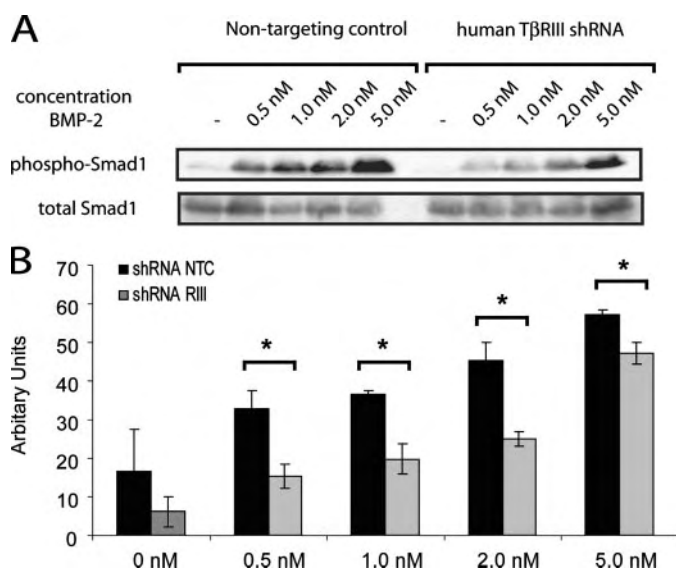


FIGURE 6. Loss of TβRIII decreases BMP-sensitivity. PC-3 cells were infected with adenovirus expressing either a non-targeting control shRNA (NTC) or shRNA directed against human TβRIII (shRIII). *A*, PC-3 cells were then treated with the indicated concentrations of BMP-2 (0, 0.5, 1.0, 2.0, and 5.0 nM, respectively) for 10 min and phospho-Smad1 and total Smad1 were detected by Western blot. Image is representative of two independent experiments. *C*, densitometry was performed on Western blot images using ImageJ. Graphed are raw densitometry units from two independent experiments for both shNTC and shRIII. Error bars represent mean \pm S.E. Two-tailed Student's *t* test was used to determine statistical significance; *, *p* < 0.05.

The differences noted between BMP ligand binding to TβRIII and the biological effect of these BMP ligands may be explained by the relative expression level of the ligands and the identity of the receptors recruited into the signaling complex. The related TGF-β superfamily co-receptor, endoglin, participates in differentially activating ALK1 and ALK5 in endothelial cells, with low TGF-β concentrations activating ALK1 and stimulating angiogenesis, whereas higher concentrations activate ALK5 to inhibit angiogenesis (56–58). The present data demonstrate that BMP-2 can cause functional recruitment of TβRIII to ALK3 consistent with a role for ALK3 in stimulating endothelial cell transformation. In contrast, TβRIII leads to enhanced BMP-7 binding to ALK6, which is not expressed in the endothelial cell model used here (50, 59). Thus, these differences in receptor expression may account for the differences in functional response between BMP-2 and the other ligands (BMP-4, BMP-7, and GDF-5) and supports a model where specific ligands may initiate the assembly of particular receptor complexes to generate diversity in biological effect.

Members of the BMP family activate cellular responses through the formation of an active signaling receptor complex containing the type I and type II receptors (8). BMP binding data presented here suggest that TβRIII does not alter the formation of this active complex. Future studies will be aimed at characterizing the effect of TβRIII on the formation, stability, and activity of the signaling complex and determine whether TβRIII alters ligand binding to the other BMP type II receptors, including ActRII and ActRIIB.

A number of BMP family members, including BMP-2, BMP-4, and BMP-7, have been associated with heart development. Here we demonstrate that TβRIII is uniquely essential

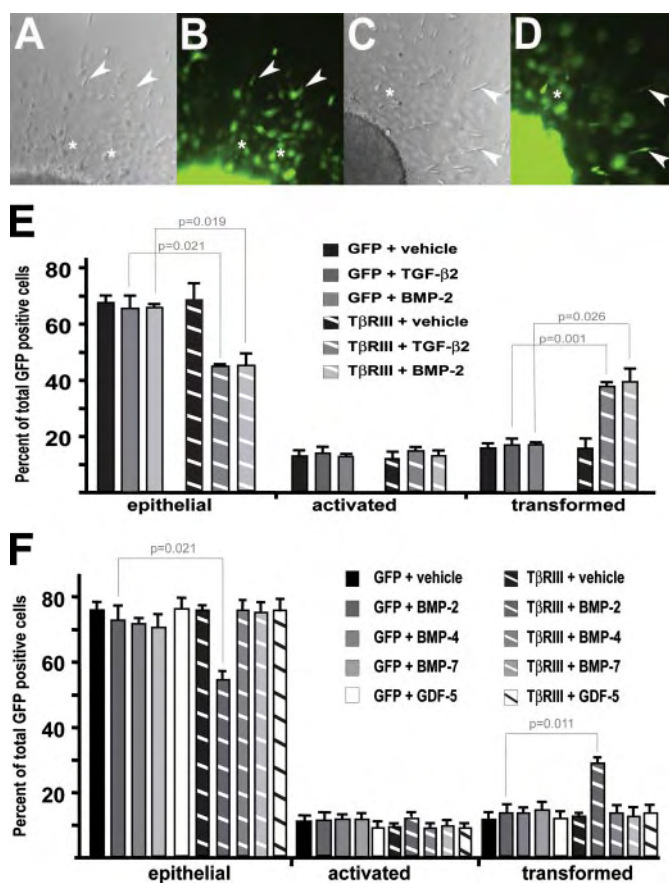


FIGURE 7. TβRIII is essential for BMP-2 and TGF-β2-mediated transformation in chick heart ventricular explants. *A–D*, chick ventricular explants infected with adenovirus expressing GFP. Photomicrographs of representative explants to illustrate the phenotypes of the infected cells. *A* and *B*, bright-field and fluorescent images with the plane of focus at the surface of the collagen pad. A sheet of rounded, adjacent cells scored as epithelial cells (asterisks) is seen adjacent to the cardiac muscle. Cells scored as activated are no longer rounded and have separated from adjacent cells but remain on the surface of the collagen pad (arrowheads). *C* and *D*, brightfield and fluorescent images with the plane of focus within the collagen pad. Epithelial cells are found on the surface (asterisk). Elongated cells in the gel are scored as transformed (arrowheads). *E*, chick ventricular explants infected with adenovirus expressing either GFP or TβRIII and GFP were incubated with either vehicle (bovine serum albumin/HCl), 200 pM TGF-β2, or 5 nM BMP-2 as indicated. *F*, TβRIII-dependent EMT is specific to BMP-2. Infected explants were incubated with 5 nM BMP-2, BMP-4, BMP-7, and GDF-5 as indicated. After incubation for 36 h, the explants were fixed and GFP-expressing cells were scored as epithelial, activated, or transformed from three independent experiments (refer to supplemental Tables S3 and S4 for actual counts). Two-tailed Student's *t* test was used to determine statistical significance.

for BMP-2-induced EMT in the developing heart, as TβRIII expression, did not confer BMP-4- or BMP-7-induced EMT. Our data are consistent with recently published data indicating that BMP-4 is dispensable for EMT in the heart (60) and that BMP-7 antagonizes TGF-β1-induced EMT, whereas having no effect on EMT by itself (61). Thus, EMT is not a physiologically relevant assay for BMP-4 and BMP-7. These data further support the hypothesis that TβRIII does not confer BMP function, but facilitates their function. Further investigation will be required to define the effect of TβRIII on other BMP family ligands in physiologically relevant assays.

Binding of BMP to TβRIII occurs through two ligand binding domains on the core protein of TβRIII, similar to TGF-β, and

does not require the heparan sulfate and chondroitin sulfate modifications to the extracellular domain of TβRIII. Whereas our data indicate that the glycosaminoglycan chains are not necessary for BMP binding, they may still contribute to enhance or alter ligand binding. The mechanisms regulating post-translational processing of TβRIII are not well understood and likely to be cell type-specific.³ The effect of differential post-translational processing on altering interactions of TGF-β superfamily members with TβRIII warrants additional investigation.

In addition to glycosaminoglycan modifications, the extracellular domain of TβRIII is proteolytically cleaved from the membrane and shed into the extracellular environment. Here we demonstrate that recombinant, purified sTβRIII can bind BMP, indicating that the interaction is direct and that anchorage to the cell membrane and proximity of other BMP binding components are not necessary for ligand binding. BMP is secreted into the extracellular environment as an active ligand and the bioavailability of BMP is tightly controlled by a number of soluble BMP antagonists that bind ligand and sequester BMP from the signaling receptors, including Noggin, Chordin, Follistatin, and gremlin (4, 7). The ability of sTβRIII to bind BMP-2 suggests that sTβRIII may be an additional mechanism by which the bioavailability of BMPs is regulated.

Expression of both BMP and TβRIII are essential during embryonic development. Here we demonstrate that TβRIII is important for BMP-induced EMT in ventricular cells of the chick heart. Therefore, defects in development of the TβRIII knock-out mice may not only be due to alterations in TGF-β signaling, but also alterations in BMP signaling. In addition to the role of BMP in development, alterations in the BMP signaling pathway have been linked to a number of hereditary human diseases, including primary pulmonary hypertension, juvenile polyposis syndrome, ovarian dysgenesis 2, and A2 brachydactyly (62, 63). BMP signaling also has an emerging role in regulating cancer biology with effects on glioblastoma (64), along with breast (65, 66), ovarian (67), pancreatic (68), colon (69), and prostate cancer cells (70). The expression of TβRIII is lost in a number of these same cancer types, including breast (33), ovarian (71), pancreatic (32), and prostate cancer (72, 73). Here we demonstrate that loss of TβRIII expression results in a decrease in cellular sensitivity to BMP, as assayed by Smad1 phosphorylation. Further defining the contribution of TβRIII to BMP signaling will aid in establishing the mechanism by which TβRIII functions during tumorigenesis, and whether alterations in TβRIII expression or function are linked to other human diseases.

Acknowledgments—We thank Tam How for technical support, TβRIIIΔgag construct generation, and adenovirus generation; Dr. Fernando Lopez-Casillas for generous donation of TβRIII constructs; members of Dr. Michael Zalutsky's laboratory for technical advice and use of equipment for radiolabeling; Millie McAdams and Dr. Munir Alam for assistance in experimental design and data analysis of BIAcore data; and Tyson Foods, Inc. and Andries Zijlstra for the generous donation of the chick eggs.

³ K. C. Kirkbride and G. C. Blobe, unpublished observations.

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Kellye Colleen Kirkbride

Home Address:

5102 Gable Ridge Dr.
Durham, North Carolina 27713
(919) 402-9338

E-mail Address: kck4@duke.edu

School Address:

213 MSRB, Campus Box #2631
Durham, North Carolina 27710
(919) 668-1353

Education:

8/01- 9/07 **Doctor of Philosophy, University Program in Molecular Cancer Biology; Certificate in Teaching College Biology (May 2006); Duke University, Durham, North Carolina.**

8/97-5/01 **Bachelor of Science with Highest Distinction (3.92/4.0 GPA), Biology; Area Certificate in Animal Behavior and minor in Psychology; Indiana University, Bloomington, Indiana.**

Laboratory Experience:

8/02-present **Doctoral Thesis Research, Duke University, Durham, North Carolina.**

- **Advisor:** Dr. Gerard C. Blobe, MD, PhD
- **Title:** "Elucidating the role of the Transforming Growth Factor- β Type III Receptor (T β RIII) in Bone Morphogenetic Protein (BMP) Signaling"
 - Identified and characterized T β RIII as a cell surface receptor for the BMP subfamily of ligands.
 - Demonstrated that T β RIII differentially alters subcellular localization of the BMP receptors, ALK3 and ALK6, through its ability to bind β -arrestin2.
 - Determined that T β RIII increases proliferation of colon cancer cells in response to BMP and TGF- β stimulation.
 - Worked in collaboration with Todd Townsend in Dr. Joey Barnett's Lab at Vanderbilt University to demonstrate that T β RIII is required for endothelial to mesenchymal transition (EndMT) in avian atrioventricular cushion formation during heart development.
- Applied for and received a Pre-doctoral Traineeship Award from the United States Department of Defense Breast Cancer Research Program (BC044590).
- Participated in a number of collaborative studies within the Blobe laboratory and with other laboratories at Duke and other research universities.
- Identified the lack of a thorough literature review on the function of growth factor co-receptors in cellular function. Contacted a peer-reviewed journal to inquire about their interest in a review and worked in conjunction with a fellow graduate student and Dr. Gerry Blobe to write the review article.
- Co-reviewed a number of submitted publications with advisor.

8/02-5/06 **Undergraduate and First Year Graduate Student Mentor, Duke University, Durham, North Carolina.**

Served as a mentor for rotation students and several undergraduate students during their tenure in our laboratory. My main responsibility was to oversee their independent research. Designed and mentored individual research projects of two senior undergraduate Duke students for 2 years.

- 8/01-7/02 Graduate Student Rotations, Duke University, Durham, North Carolina.**
During my first year as a graduate student, I spent approximately 3 months in each of the following laboratories: Dr. Perry Blackshear (National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina) and Dr. Robert Wechsler-Reya (Duke University).
- 8/01-5/03 Graduate Student Coursework, Duke University, Durham, North Carolina.**
Courses complete include: Cellular Signaling, Fundamentals of Human and Mouse Genetics, Modern Techniques in Molecular Biology, Molecular Cell Biology, Molecular Mechanisms of Oncogenesis, Physical Chemistry for Biologist and Structure of Biological Macromolecules.
- 1/00-5/01 Undergraduate Research in Biology, Indiana University, Bloomington, Indiana.**
Conducted research under the guidance of principle investigator, Dr. Wayne Forrester. Obtained the skills necessary to perform a variety of laboratory techniques dealing with *C. elegans*, including vector constructions, PCR, ligation and cloning procedures. My project included the generation of transgenic animals and a mutagenesis screen.
- Teaching Experience:**
- 8/04-8/06 Peer Tutoring Program, Duke University, Durham, North Carolina.**
Tutored students in Introductory General Chemistry, which allowed me to become familiar with different learning styles and work on communicating difficult concepts and how to solve problems in the course.
- 1/04-5/04 Teaching Assistant, Duke University, Durham, North Carolina.**
Served as the primary instructor for an Introductory Microbiology Laboratory course. Presented small introductory lectures and aided the students in hands on scientific research. Responsibilities included: preparing weekly quizzes, midterm and final exams, along with grading lab reports.
- 8/03-5/04 Preparing Future Faculty Program, Duke University, Durham, North Carolina.**
Participated in a year long program that introduced graduate students to teaching and academic environment outside of institutions similar to Duke University. Visited and talked to instructors at Guildford College, Meredith College, Elon University and North Carolina Central University. Through this program, I was mentored by Dr. Elizabeth Wolfinger at Meredith College. Participated in several teaching workshops.
- 6/02, 1/03-5/03 Guest Lecturer, Durham Technical Community College, Durham, North Carolina.**
Served as a teaching assistant/guest lecturer for a general biology laboratory course. Prepared weekly quizzes, small lectures on background information and helped students to successfully apply the classroom information to biologically relevant personal experiences.

8/99-12/99 Undergraduate Teaching Intern, Department of Biology, Bloomington, Indiana.

Led a discussion section of 20 students for an upper level Genetics course. I was responsible for carrying out many of the administrative responsibilities of teaching a class, including worksheet, quiz and exam question development and grading.

Publications:

1. **Kirkbride KC**, Townsend TA, Bruinsma MW, Barnett JV and Blobe GC. *Bone Morphogenetic Proteins signal through the Transforming Growth Factor- β Type III Receptor*. **Journal of Biological Chemistry** (2008) March; **283** (12):7628-37.
2. Gordon KJ*, **Kirkbride KC*** and Blobe GC. *Bone Morphogenetic Proteins induce pancreatic cancer cell invasion through MMP-2*. (Manuscript in preparation) (*contributed equally)
3. **Kirkbride KC***, Lee NY*, Sheu R and Blobe GC. *The Transforming Growth Factor- β Type III Receptor Mediates Differentially Internalization of the BMP Type I Receptors to Alter Signaling*. (Manuscript in preparation) (contributed equally)
4. **Kirkbride KC**, Serwer LP, Sheu RD*, Dong MD*, You HJ*, How T*, Fields T and Blobe GC. *The Transforming Growth Factor- β Type III Receptor enhances colon cancer proliferation*. (Manuscript in preparation). (*contributed equally)
5. Dong M, How T*, **Kirkbride KC***, Gordon KJ*, Lee JD*, Hempel N*, Kelly P, Moeller BJ, Marks J and Blobe GC. *The type III TGF- β Receptor suppresses breast cancer progression*. **Journal of Clinical Investigation** (2007) Jan; **117**(1):206-217. (*contributed equally)
6. *ALK3 Molecule Page*. The Alliance for Cellular Signaling (Nature). Accepted for publication (February 2006) www.signaling-gateway.org/molecule/query?afcsid=A000256
7. **Kirkbride KC***, Ray BN* and Blobe GC. *Cell Surface Co-Receptors: Emerging Roles in Signaling and Human Disease*. **TRENDS in Biochemical Sciences** (2005) Nov; **30**(11):611-621. (*contributed equally)
8. *TGF-beta Mini-molecule web page*. The Alliance for Cellular Signaling (Nature). www.signaling-gateway.org/molecule/query?type=mmpdocs&afcsid=A002271&mpv=current
9. Chen W, **Kirkbride KC**, How T, Nelson CD, Frederick JP, Mo J, Wang XF, Lefkowitz RJ, and Blobe GC. *β -Arrestin2 Mediates Internalization of the Type III TGF- β Receptor and Down Regulation of its Signaling*. **Science** (2003) **301**: 1394-1397.
10. **Kirkbride KC** and Blobe GC. *Inhibiting Transforming Growth Factor- β as a Means of Immunotherapy*. **Expert Opinion in Biological Therapy** (2003) Apr; **3**(2):251-61.

Presentations:

Posters:

- **Kirkbride K**, Mo J, and Blobe GC. **“Role of the Type III TGF- β Receptor in Mediating TGF- β Superfamily Signaling”**- Department of Pharmacology and Cancer Biology Annual Retreat (October 2002); 11th Annual Biological Sciences Graduate Student Symposium (November 2002).
- Chen W, **Kirkbride KC**, How T, Nelson CD, Mo J, Frederick JP, Wang XF, Lefkowitz RJ and Blobe GC. **“ β -arrestin2 Mediates Endocytosis of the Type III TGF- β Receptor and Down-regulation of its signaling”**- FASEB Summer Research Conference (July 2003); 12th Annual Biological Sciences Graduate Student Symposium (November 2003).
- **Serwer L**, **Kirkbride KC**, Elliott RL and Blobe GC. **“Smad-Independent Transforming Growth Factor- β Signaling”** (Howard Hughes Undergraduate Summer Research -August 2003).
- **Elliott RL**, **Kirkbride KC** and Blobe GC. **“Transforming Growth Factor beta induced MAP kinase activation.”**- Department of Pharmacology and Cancer Biology Annual Retreat (October 2003).
- **Sheu R**, **Kirkbride KC** and Blobe GC. **“The Role of the Transforming Growth Factor- β Type III Receptor on Gene Expression in Human Breast Cancer Cells”** Howard Hughes Summer Research Programs Poster Session (August 2004) and Duke University Visible Thinking: A Presentation of Undergraduate Research (April 2005).
- **Kirkbride KC** and Blobe GC. **“Role of the Type III Transforming Growth Factor- β Receptor (T β RIII) in bone morphogenetic protein signaling”** Department of Pharmacology and Cancer Biology Annual Retreat (October 2004)
- **Sheu R**, **Kirkbride KC** and Blobe GC. **“The Role of the Transforming Growth Factor- β Type III Receptor in Bone Morphogenetic Protein Signaling.”** Howard Hughes Summer Scholars Research Programs Poster Session (August 2005).
- **Dong M**, How T, **Kirkbride KC**, Gordon KJ, Kelly P, Moeller, Hempel N, Marks JR and Blobe GC. **“The type III TGF- β receptor suppresses breast cancer progression.”** Department of Pharmacology and Cancer Biology Annual Retreat (September 2005); AACR Special Conference- TGF- β in Cancer and Other Diseases (February 2006).
- **Kirkbride KC**, Sheu RD, Blobe GC. **“The Type III Transforming Growth Factor- β Receptor mediates Bone Morphogenetic Protein Signaling.”** (1) Department of Pharmacology and Cancer Biology Annual Retreat (September 2005) and (2) Biological Sciences Graduate Student Symposium (November 2005).
- **Kirkbride KC**, Sheu RD, Blobe GC. **“The Transforming Growth Factor- β Type III Receptor (Betaglycan) is a Co-receptor for the Bone Morphogenetic Proteins”** AACR Special Conference- TGF- β in Cancer and Other Diseases (February 2006).

- Sheu RD, Kirkbride KC, Blobe GC. “**Transforming Growth Factor- β Type III Receptor plays a role in Bone Morphogenetic Protein Signaling**” Visible Thinking- A Presentation of Undergraduate Research (April 2006).
- Serwer LP, Kirkbride KC, Blobe GC. “**Loss of Transforming Growth Factor- β Receptor Type III in Human Colorectal Cancer**” Visible Thinking- A Presentation of Undergraduate Research (April 2006).
- Kirkbride KC and Blobe GC. “**Betaglycan: a co-receptor for the Bone Morphogenetic Proteins and its involvement in Colon Cancer**” 15th Annual Biological Science Graduate Student Symposium (September 2006).
- Kirkbride KC, Sheu RD, Blobe GC. “**The Transforming Growth Factor- β Type III Receptor (Betaglycan) is a Co-receptor for the Bone Morphogenetic Proteins**” (1) Gordon Research Conference-Proteoglycans (July 2006); (2) Biological Sciences Graduate Student Symposium (November 2006);
- Kirkbride KC, Sheu RD, Serwer LP, Dong M, Bruinsma MW and Blobe GC. “**Characterizing Betaglycan as a co-receptor for the Bone Morphogenetic Proteins and Involvement in Colon Cancer**” Duke University Comprehensive Cancer Center Conference (March 2007).

Oral:

- Kirkbride KC, Chen W, How T, Nelson CD, Mo J, Frederick JP, Bruinsma M, Wang XF, Lefkowitz RJ and Blobe GC. “ **β -arrestin2 Mediates Endocytosis of the Type III TGF- β Receptor and Down-regulation of its signaling**”- Duke University Department of Pharmacology and Cancer Biology Annual Departmental Retreat (October 2003).
- Kirkbride KC, Sheu RD and Blobe GC. “**The Transforming Growth Factor- β Type III Receptor (Betaglycan) is a co-receptor for the Bone Morphogenetic Proteins**” –Sixth Annual Graduate Student Research Day, Duke University (April 2006)
- Kirkbride KC, Bruinsma M, Dong M, Finger E, Gordon K, Hempel N, How T, Lee J, Sheu R, Turley R and Gerard C. Blobe. “**The Transforming Growth Factor- β Type III Receptor: a Co-receptor for the Bone Morphogenetic Proteins and its Involvement in Cancer Progression.**” Duke University Department of Pharmacology and Cancer Biology Annual Retreat (September 2006)

Awards and Honors:

- Department of Defense- Breast Cancer Research Predoctoral Training Grant Recipient (2005-2008)
- Conference Travel Fellowship, Duke University Graduate School (AACR Meeting) (2006)
- Duke University Center for Teaching, Learning and Writing Mini-grant Recipient (2003)
- National Science Foundation Graduate Research Fellowship- Honorable Mention (2003)
- Molecular Cancer Biology Training Grant (2001-2003)
- Phi Beta Kappa (2000)

Professional Societies:

- American Association for Cancer Research
- American Association for the Advancement of Science

Technical Skills:

Adenoviral work
Alkaline phosphatase differentiation assays
Antibody verification
Biotinylation (cell surface)
Caspase assays using Promega Caspase-GLO reagent
Chemical crosslinking of radiolabeled ligands to cell surface receptors
Extraction and purification of DNA and RNA
Gelatin zymography
 Immunoblotting
 Immunofluorescence
 Immunohistochemistry
 Immunoprecipitation (co-immunoprecipitation)
 Internalization assays using radiolabeled ligand
 Luciferase reporter assays
 Mammalian cell culture

Matrigel invasion assay
Microscope work (basic)
Mouse work- basic techniques including:
harvesting organs, measuring tumor volumes
Mutagenesis
Orthophosphate labeling
Polymerase Chain Reaction (PCR)
Radiolabeling of proteins
Real-time PCR
Reverse transcription-PCR
RNAi development
Soft Agar colony formation assays
Thymidine incorporation
Transfection
Transwell motility assays
Wound closure assays

References:

References available upon request.